REMARKS

Claim 1 has been amended to specify that the determination is made by nucleic acid analysis. Claim 1 has further been amended to indicate that the person belongs to a population that is heterozygous for the specified polymorphism. Support for this latter amendment can be found, for example, at page 17, lines 14-24.

Claim 3 has been canceled without prejudice to pursuing in a continuation application.

Claim 10 has been canceled in view of the amendment of claim 1.

It is submitted that none of the above amendments constitute new matter and their entry is requested.

The Examiner has rejected claims 1 and 3 under 35 U.S.C. § 112, first paragraph for lack of enablement. In essence, this rejection consists of two parts. First, the Examiner contends that the specification does not enable methods for the detection of the polymorphism by the use of specific antibody or other polypeptide analysis. Second, the Examiner contends that the specification is not enabling with respect to the sufficiency of the data presented in the specification with respect to the association between the claimed polymorphism.

Although Applicants do not agree with the Examiner's contention that protein analysis methods are not enable, they have canceled this subject matter in order to expedite the allowance of the present application and without prejudice to filing a continuation application to pursue such subject matter.

Applicants submit that the Examiner is in error with respect to the sufficiency of the data in the specification and that this data supports the association between the claimed polymorphism and alcoholism. Specifically, Applicants offer the following comments concerning the data presented in the specification.

In the initial written description Applicants have demonstrated that the proline7 substitution is associated with an elevated consumption of alcohol. Presence of proline7 was associated with an approximately one-third (33%) higher average consumption of pure alcohol as compared to homozygous subjects with the leucine7/leucine7. This novel finding is very remarkable, since the

discovery has been made in a population based sample of individuals. Discovering a single gene effect in a population based sample is very significant, because it is a commonly recognised fact that alcohol consumption is determined by several inherited (i.e. genetic) and environmental factors. In addition, clinically alcoholism is not a single entity but a group of diseases with several polygenic quantitative traits, which may have variable aetiology in different patients. In a population based sample, which is not enriched for desired disease state (in this case alcoholism), a single gene effect on alcohol consumption may be even impossible to determine, but if it can be discovered, it is probably stronger than the initial finding allows estimating. This finding of the association of NPY system (leucine7/proline7 polymorphism in the preproNPY) with alcohol consumption in human, is however, fully supported by animal experimentation, which has been reviewed in detail in our application.

Based on the initial written description, Applicants made a discovery that the carriers of an allele associated with proline7 substitution would have higher likelihood of becoming alcoholics and this novel finding was subjected for patent application process. In the written description, Applicants have described the proportion of heavy consumers of alcohol (defined by the use of more than 230 g of pure ethanol per week, which is considered an amount to qualify for alcoholism) also higher among the carriers of proline7 substitution (13.1% vs. 8.2%) when compared to the non carriers of proline7 (see description, page 16, lines 5-9). In the statistical analyses, chi-squared test estimated the probability of difference significant at p=0.10 level. Statistical significance level was adjusted at p=0.05, which is a common requirement for several scientific papers. As discussed in the application p=0.05 level of significance was not reached due to small numbers of subjects with heavy user status (this is why population based samples may underestimate the effect of the single gene and the significance of the discovery). However, p-value is only a probability for estimating the significance of the difference, not the probability of the observed difference between the means. Sometimes probability level p=0.10 or p=0.01 is used instead of p=0.05 level for significance. Thus, if the significance of the difference does not reach p=0.05 level, it does not mean that the difference does not exist. The observed difference (13.1% vs. 8.2%) is a discovery that clearly demonstrates

the working, usefulness and inventive step of the invention, which thus fulfils the requirements for patenting. This initial discovery was demonstrated to be true in another, indeed very carefully designed and sophisticated U.S. study of the association of proline7 substitution and alcoholism, namely the study by Lappalainen et al. ("A functional neuropeptide Y Leu7Pro polymorphism associated with alcohol dependence in a large population sample from the United States." *Arch Gen Psychiatry* 2002 Sep;59(9):825-31) (cited by the Examiner) that fully supports Applicants' initial invention that the proline7 substitution is associated with alcohol consumption and alcoholism. Therefore, Applicants kindly submit that this description demonstrates that there is an association of proline7 substitution with alcoholism and alcohol consumption, which qualifies for patenting.

In response to questions raised by the Examiner, Applicants offer the following answers.

It is obvious, natural and even expected that the results of scientific research diverge. Although the study settings are the same, sometimes the result is different and affected by several uncontrollable factors. This may be due to errors made or by a change. However, from patenting aspect, divergent results should not affect the initial discovery and application, in which the invention is described in a way that a person skilled in the art is able to reproduce it. Indeed results presented post filing date support our initial finding and the association of proline7 with alcoholism as discussed previously. There are two positive, accordant reports of an association of proline7 with alcoholism by Kauhanen et al. ("Neuropeptide Y polymorphism and alcohol consumption in middle-aged men." *Am J Med Genet.* 2000 Jul 17;93(2):117-21) (copy attached) and Lappalainen et al. in comparison to one no-association report by Zhu et al. ("NPY leu7pro and alcohol dependence in Finnish and Swedish populations." *Alcohol Clin Exp Res.* 2003 Jan;27(1):19-24) (cited by Examiner). In other studies gene polymorphism responsible for proline7 was not detected, and the association of proline7 with alcoholism was not investigated. The study by Ilveskoski et al. ("Association of neuropeptide y polymorphism with the occurrence of type 1 and type 2 alcoholism." *Alcohol Clin Exp Res.* 2001 Oct;25(10):1420-1422) (cited by Examiner) can not be considered representative for comparison for the reasons presented below. Therefore, although post filing date

presented results should not contradict application process, after critical review, the overall result more supports than questions Applicants' discovery.

The study referred by the examiner of Ilveskoski et al. is unfortunately biased. Therefore it is not relevant to refer to it. Specifically, the sample of alcoholics consists of only 122 subjects. In addition, in the control population, which is only of 59 subjects and far too small for an association study, the carrier frequency of proline 7 substitution was reported to be 24.1%, which is roughly doubled to that of reported in Finish population in general (Kauhanen et al. and Karvonen et al., "Association of a leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels." *Nat Med.* 1998 Dec;4(12):1434-7) (attached is a copy of the latter for which an abstract was previously cited in an Information Disclosure Statement). The first rule in all association studies is that the control population is representative, and that the genotype distribution follows Hardy-Weinberg equilibrium (binomial square). Therefore, the reported frequencies in the study by Ilveskoski et al. raises concerns of errors in genotyping such as contamination or errors in sampling. Therefore, the authors of the study by Ilveskoski et al. have made biased conclusions that the genetic polymorphism producing the proline7 substitution of preproNPY might not predispose to alcoholism, but indeed retard the transition to alcoholism.

Drube et al. screened 105 Japanese patients with alcoholism (Drube et al., "No leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y in Japanese population or Japanese with alcoholism." *Psychiatr Genet.* 2001 Mar;11(1):53-5) (cited by Examiner) and did not find the genotypes associated with proline7 polymorphism. This finding does not exclude a possibility that the Pro7 substitution is associated with alcoholism or alcohol consumption in a Japanese population. 105 patients represent a minor proportion of Japanese alcoholics, and can not therefore be considered as a representative sample of all Japanese alcoholics. It is also probable that although not originally present in the Japanese population ancestry, proline7 is or will be penetrated in Japanese heritage. This invasion of genes/gene polymorphism is genetically very natural and well recognized phenomenon. Further, we have demonstrated that the proline7 substitution is associated with alcohol

consumption, not that the proline7 substitution is present in Japanese population. Therefore, as simple as it is, proline7 substitution can not be associated with alcohol consumption if it is not detected, and the non-predictable value of proline7 substitution remains to be demonstrated in Japanese population. Importantly, it should not prevent the patenting of the initial demonstrated findings.

Zhu et al. have made a meta-analysis of the frequencies of the proline7 polymorphism, not of the association of proline7 polymorphism with alcoholism. However, they haven't carefully enough studied the paper by Ilveskoski et al., which is biased. Therefore it is not relevant to refer to, and also some of the conclusions made by Zhu et al. are not relevant.

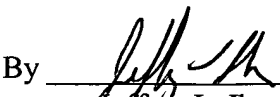
Although Applicants do not believe that Drube et al. negatively impacts the sufficiency of the data demonstrating an association between the claimed polymorphism and alcoholism, they have nevertheless amended the claims to indicate that this association is detected in a patient in a population that is heterozygous for the claimed polymorhism.

In view of the amendments of the claims and the above remarks, it is submitted that the present application enables the claimed invention. Withdrawal of this rejection is requested.

In view of the above amendments and remarks, it is submitted that the claims satisfy the requirements of the patent statutes and are patentable over the prior art. Reconsideration of the instant application and early notice of allowance are requested. The Examiner is invited to telephone the undersigned if it is deemed to expedite allowance of the application.

Respectfully submitted,

ROTHWELL, FIGG, ERNST & MANBECK, p.c.

By 

Jeffrey L. Ihnen
Registration No. 28,957
1425 K Street, N.W., Suite 800
Washington, D.C. 20005
Telephone No.: (202) 783-6040
Facsimile No.: (202) 783-6031

Attachments: Karvonen, et al. Association of a leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels. Nat Med. 1998 Dec;4(12):1434-7.

Kauhanen, et al. Neuropeptide Y polymorphism and alcohol consumption in middle-aged men. Am J Med Genet. 2000 Jul 17;93(2):117-21

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Association of a leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels

MATTI K. KARVONEN[1], ULLAMARI PESONEN[1], MARKKU KOULU[1], LEO NISKANEN[2], MARKKU LAAKSO[2], AILA RISSANEN[3], JACQUELINE M. DEKKER[4], LEEN M 'T HART[5], RAISA VALVE[6] & MATTI I.J. UUSITUPA[6]

[1]*Department of Pharmacology and Clinical Pharmacology, University of Turku, Kiinamyllynkatu 10, FIN-20520 Turku, Finland*

[2]*Department of Medicine, University of Kuopio, P.O.Box 1777, FIN-70211 Kuopio, Finland*

[3]*Eating Disorder Unit, University of Helsinki, Paasikivenkatu 4, FIN-00250 Helsinki, Finland*

[4]*Institute for Research in Extramural Medicine, Vrije Universiteit Amsterdam, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands*

[5]*Leiden University Medical Center, Department of Molecular Cellbiology, Wassenaarseweg 72, 2333 AL, Leiden, The Netherlands*

[6]*Department of Clinical Nutrition, University of Kuopio, P.O.Box 1627, FIN-70211 Kuopio, Finland*

Correspondence should be addressed to M.K.; email:mkoulu@utu.fi

High serum levels of total and LDL cholesterol are important risk factors in the development of atherosclerotic coronary artery disease. Cholesterol metabolism is affected by nutritional, environmental and genetic factors. Neuropeptide Y (NPY), which is widely expressed in both the central and peripheral nervous systems[1,2], has an important role in the hypothalamic regulation of energy balance by stimulating food intake[3–5] and favoring energy storage through increased lipoprotein lipase activity in white adipose tissue[6,7]. As a part of ongoing study of the genetic basis of obesity, we screened the *NPY* gene for sequence variants. We report here the identification of a common Leu(7)-to-Pro(7) polymorphism in the signal peptide of NPY. Presence of this Pro(7) in NPY was associated with higher serum levels of total and LDL cholesterol in obese subjects participating in two independent Finnish and Dutch studies. Furthermore, normal-weight Finns with Pro(7) also had higher serum levels of total and LDL cholesterol than did subjects with Leu(7)/Leu(7), as analyzed in three subsequent determinations at 5-year intervals during a 10-year follow-up period. The NPY polymorphism was not associated with higher cholesterol levels in normal-weight Dutch. Our study provides evidence that *NPY* is linked to cholesterol metabolism and that the polymorphism producing Pro(7) in NPY is one of the strongest genetic factors identified thus far affecting serum cholesterol, particularly in obese subjects.

We screened the entire coding region of the *NPY* gene using single-stranded conformational analysis (SSCA). We identified a thymidine(1128)-to-cytosine(1128) polymorphism (T1128C), which results in a substitution of Leu(7) by Pro(7) in the signal peptide part of pre-pro-NPY. We first analyzed the effect of the Leu(7)-to-Pro(7) polymorphism on obesity and metabolic parameters in obese, non-diabetic Finns (n = 138), and found strong associations with high serum total and LDL cholesterol levels, which were subsequently confirmed in normal-weight Finns (n = 64). Then we analyzed a sample of a cohort study from The Netherlands including both obese (n = 93) and normal-weight (n = 263) subjects to confirm the role of the NPY polymorphism in a population with different genetic background. All subjects were genotyped for the polymorphism using the restriction fragment length polymorphism method, based on the *Bsi*EI restriction enzyme recognition site. The C1128 allele frequency was similar in obese and normal-weight subjects in both populations. There was, however, a difference in the allelic frequencies between the Finns and the Dutchmen (0.08 and 0.03, respectively; $\chi^2 = 8.36$; $P < 0.01$).

Among the genotyped Finnish obese subjects, one was homozygous for the polymorphism producing Pro(7)/Pro(7); she was included in the 'heterozygous Leu(7)/Pro(7)' group for the association analysis. When compared with subjects with Leu(7)/Leu(7) (n = 118), the subjects with Pro(7) (n = 20) had significantly higher levels of serum total cholesterol (6.2 ± 1.1 compared with 5.3 ± 0.9 mmol/l, mean ± s.d.; P = 0.0001, Student's t-test; 95% confidence interval (CI) for the difference of the means from 0.44 to 1.34 mmol/l) and LDL cholesterol (4.3 ± 1.0 compared with 3.5 ± 0.8 mmol/l; P = 0.0001; 95% CI, 0.41–1.20) (Fig. 1*a*). These differences were also statistically highly significant after adjustments for age and sex (P = 0.0001 for the genotype differences in both serum total and LDL cholesterol levels). We analyzed the probability of having Pro(7) with respect to serum total cholesterol level in obese Finns, using a multiple linear regression model (Fig. 2). In further analyses, subjects with Pro(7) were found to have slightly lower levels of serum HDL cholesterol than subjects with Leu(7)/Leu(7) (1.04 ± 0.28 compared with 1.20 ± 0.29 mmol/l; P = 0.029; 95% CI, 0.016–0.29). Serum VLDL cholesterol, triglyceride, distribution of the apolipoprotein E phenotypes, intake of total fat, saturated fatty acids, unsaturated fatty acids or dietary cholesterol (based on diet diary data) did not differ between the *NPY* genotype groups in obese Finns (data not shown). Having Pro(7) did not produce any effect on obesity or parameters related to energy metabolism, including body weight, body mass index, waist-to-hip ratio, basal metabolic rate or respiratory quotient (Table).

Our investigation of the role of the *NPY* polymorphism in obese Dutch subjects confirmed our initial observation made in obese Finns. When compared with subjects with Leu(7)/Leu(7) (n = 86),

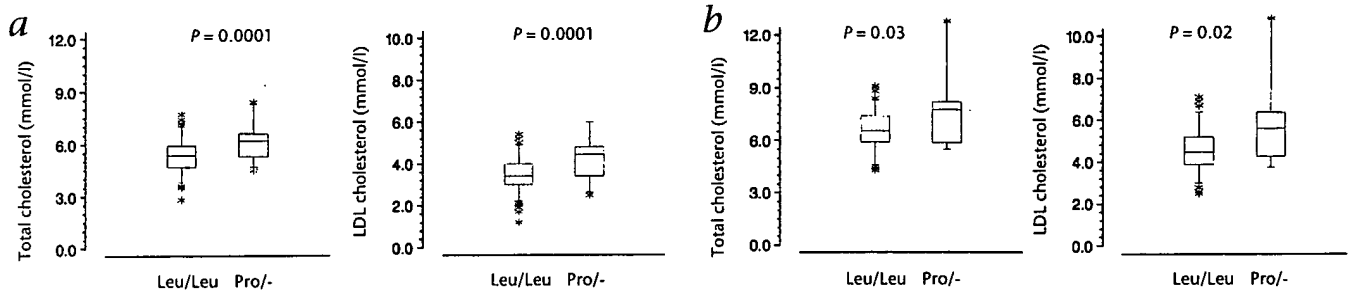

Fig. 1 Fasting serum total cholesterol and LDL cholesterol levels in obese Finns (***a***) and obese Dutchmen (***b***). The box plots represent the 25th, 50th (median) and 75th percentiles of the given parameter; the 5th and 95th percentiles are small horizontal lines above and below the box plots. Extreme observations are marked by asterisks. Leu/Leu, subjects with Leu(7)/Leu(7); Pro/–, subjects with either Leu(7)/Pro(7) or Pro(7)/Pro(7).

subjects with Pro(7) (n = 6, all heterozygous) had significantly higher levels of serum total cholesterol (8.0 ± 2.6 compared with 6.6 ± 1.1 mmol/l; P = 0.034, Student' t-test; 95% CI for the difference of the means from 0.35–2.46) and LDL cholesterol (6.1 ± 2.5 compared with 4.6 ± 1.0 mmol/l; P = 0.016; 95% CI, 0.53–2.47) (Fig. 1*b*). After adjustments for age and sex, the differences were statistically highly significant (P = 0.005 for the difference in serum total cholesterol; P = 0.001 for the difference in serum LDL cholesterol).

We also analyzed the effect of Pro(7) in NPY on normal-weight Finnish and Dutch subjects. In 64 normal-weight Finns (body mass index <27 kg/m^2), subjects with Pro(7) (n = 8; including one with a homozygous genotype for this polymorphism) had, during the 10-year follow-up period, consistently higher levels of serum total cholesterol (7.5 ± 0.6 compared with 6.7 ± 0.9 in 1979–81; 7.3 ± 0.6 compared with 6.7 ± 1.3 in 1985–86; and 7.6 ± 1.0 compared with 6.4 ± 0.8 mmol/l in 1991–92; genotype effect F = 7.28, P = 0.009; time effect F = 0.91, P = 0.92; genotype × time interaction F = 0.30, P = 0.30; ANOVA for repeated measurements) and LDL cholesterol (5.2 ± 0.7 compared with 4.5 ± 0.9, 4.9 ± 0.4 compared with 4.2 ± 1.0 and 5.2 ± 0.9 compared with 4.2 ± 0.7 mmol/l, respectively; genotype effect F = 8.77, P = 0.004; time effect F = 0.39, P = 0.40; genotype × time interaction F = 0.68, P = 0.690) than did normal-weight subjects with Leu(7)/Leu(7). One subject with Pro(7) was male; if the female subjects were analyzed separately, the genotype differences in serum total and LDL cholesterol levels were still statistically significant (data not shown). The NPY genotype was not associated with changes in serum HDL cholesterol, VLDL cholesterol or triglyceride levels in normal-weight Finns. Serum apolipoprotein B levels, determined only once during the study, were significantly higher in normal-weight Finnish subjects with Pro(7) than in subjects with Leu(7)/Leu(7)(1.27 ± 0.25 compared with 1.04 ± 0.23 g/l; P = 0.003; 95% CI, 0.09–0.41 for the difference). In contrast to normal-weight Finns, the NPY polymorphism was not associated with changes in serum total cholesterol (6.5 ± 1.1 compared with 6.6. ± 1.1, not significant) or LDL cholesterol (4.6 ± 1.0 compared with 4.6 ± 1.0; not significant) levels in normal-weight Dutch subjects in the cohort studied (n = 263 total; 19 heterozygous for the polymorphism).

Here we report the identification of a newly discovered Leu(7)-to-Pro(7) polymorphism in the signal peptide part of NPY. This polymorphism was significantly and consistently associated with high serum total and LDL cholesterol levels in obese Finnish and Dutch subjects. The C1128 allele (Pro(7)) was detected in 14% of Finns and in 6% of Dutchmen. The associations were not explained by confounding factors known to affect cholesterol metabolism, such as age, sex, degree of obesity, smoking or medication. Furthermore, apolipoprotein E4 phenotypes (determined only in Finns) were evenly distributed among the *NPY* genotype groups. It is also very unlikely that the association could be due to a stratification error in sampling, because Finns are genetically a homogenous population, and because the observation was repeated in obese Dutchmen with a different genetic background. Serum total cholesterol levels were on average 0.9 mmol/l higher, and serum LDL cholesterol levels, 0.7 mmol/l higher, in obese Finns with Pro(7) than in subjects with Leu(7)/Leu(7); these differences were even more pronounced in obese Dutchmen (1.4 mmol/l and 1.5 mmol/l, respectively). The effect of this gene variant on serum cholesterol level is thus greater in obese subjects than that of the apolipoprotein E4 allele[8,9]. Therefore, the T1128C to C1128 in *NPY*, producing a Leu(7)-to-Pro(7) polymorphism, should be considered as an important genetic factor for high serum total and LDL cholesterol levels, particularly in obese subjects.

The effect of the C1128 allele in *NPY* on cholesterol levels was not as consistent in normal-weight subjects, as a significant genotype effect was found only in Finns. The conflicting results may reflect the possibility that Pro(7) in NPY has a more important role in the obese phenotype than in the lean one. This could be due to well-known abnormalities in lipid metabolism in obesity, such as considerably increased cholesterol turnover or abnormalities of LDL kinetics. As the influence of having Pro(7) on serum total and LDL cholesterol levels was significant

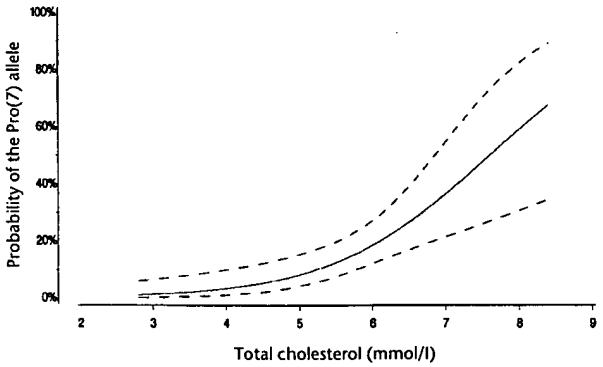

Fig. 2 Predicted probability (solid line) with 95 % confidence limits (dashed line) of having Pro(7) in NPY, calculated by logistic regression analysis with respect to serum total cholesterol level in obese Finns. The odds ratio of having Pro(7) was 2.5 (95% CI, 1.5–4.3; P < 0.001) for an increase of 1 mmol/l in serum total cholesterol. With 5.0 mmol/l, 5.5 mmol/l or 6.0 mmol/l as the upper limits of normal serum total cholesterol level, the odds ratios of having Pro(7) were 3.3 (1.07–10.6), 3.6 (1.3–10.0) and 5.9 (2.2–15.8), respectively, for subjects with elevated serum cholesterol.

Table Demographic and clinical characteristics of obese and normal-weight study subjects according to the population and NPY genotype

	Obese subjects				Normal-weight subjects			
	Finns		Dutchmen		Finns*		Dutchmen	
	Without Pro(7)	With Pro(7)	Without Pro(7)	With Pro(7)	Without Pro(7)	With Pro(7)	Without Pro(7)	With Pro(7)
N	118	20	87	6	56	8	244	19
Age, years	40.7 ± 6.1	41.9 ± 7.7	61.6 ± 8.1	65.9 ± 5.5	53.4 ± 5.6	53.5 ± 4.5	61.2 ± 7.6	62.5 ± 7.2
BMI, kg/m²	34.7 ± 3.8	35.4 ± 3.0	29.9 ± 1.6	30.3 ± 1.7	24.1 ± 1.8	25.0 ± 1.9	24.5 ± 1.9	24.5 ± 1.5
Waist-to-hip ratio	0.93 ± 0.08	0.94 ± 0.08	0.91 ± 0.08	0.94 ± 0.11	0.87 ± 0.08	0.84 ± 0.05	0.88 ± 0.08	0.87 ± 0.07
Fasting insulin, pmol/l	94.8 ± 45.3	97.7 ± 53.5	96.5 ± 37.6	100.6 ± 27.7	74.9 ± 47.2	95.3 ± 23.8	71.2 ± 24.7	64.4 ± 21.8
Fasting glucose, mmol/l	5.5 ± 0.7	5.5 ± 0.8	5.6 ± 0.7	5.4 ± 0.3	4.9 ± 0.6	4.5 ± 0.6	5.4 ± 0.5	5.2 ± 0.4

The values are mean ± s.d. The subjects without Pro(7) and with Pro(7) did not differ statistically in any of these phenotype parameters when comparing genotypes in Finnish or Dutch populations in the given subject category. *Measured at the 5-year examination.

and constant over a 10-year follow-up period in normal-weight Finns, but not in Dutchmen, a different genetic background, and environmental factors such as differences in diet, may modify the role of NPY and Pro(7) in lipid metabolism.

The Leu(7)-to-Pro(7) polymorphism is located in the signal peptide part of pre-pro-NPY. Signal peptides target nascent proteins to the endoplasmic reticulum, where proteins fold and oligomerize, disulfide bonds are formed and N-linked oligosaccharides are added. A well-defined consensus sequence for all signal peptides is not evident, but they share three common structural features: the amino-terminal part of the signal peptide contains at least one positively charged residue, there is a central hydrophobic region, and there is a carboxy-terminal region with small, nonpolar amino acids at positions –1 and –3. Leucine, which has a hydrophobic aliphatic side chain, is known to favor formation of α-helices. Proline, which has a cyclic structure that considerably influences protein secondary and tertiary architecture, does not favor formation of α-helices, but instead introduces breaks and kinks into α-helical parts of the peptide backbone. Because of these chemical differences between proline and leucine, the substitution of Pro(7) for Leu(7) in the signal peptide may lead to alterations in cellular processing of the nascent pre-pro-NPY in the endoplasmic reticulum, which could lead to changes in the availability or kinetics of NPY release. However, further studies are needed to elucidate these mechanisms in detail.

The mechanisms contributing to the association of Pro(7) in NPY to cholesterol metabolism are not known. Because there were no constant abnormalities in serum VLDL cholesterol or triglyceride levels in the affected subjects, the main defect is not likely to be in the synthesis or the catabolism of VLDL. Centrally administered NPY increases the expression of lipoprotein lipase mRNA and enhances the enzyme activity in white fat favoring lipid storage[6,7]. Thus, the possibility that the Leu(7)-to-Pro(7) polymorphism in NPY could lead to changes in lipoprotein lipase activity can not be totally excluded. The most plausible explanation for the elevation of serum cholesterol levels, however, is impaired activation of LDL receptors, which are known to regulate serum concentrations of LDL, and to a lesser degree, of IDL and VLDL particles. Apolipoprotein B is the chief protein constituent of VLDL and its metabolic products, IDL and LDL, and also serves as a ligand for LDL-receptor-mediated endocytosis of LDL (ref. 10). In normal-weight Finns with Pro(7), serum apolipoprotein B levels were significantly higher than in subjects having the Leu(7)/Leu(7) genotype. This may be due to a downregulation of LDL-receptors, which decreases the clearance of particles containing apolipoprotein B.

Connections between NPY and serum cholesterol levels have been investigated in only one earlier study, which reported that plasma concentrations of NPY correlated positively with serum cholesterol levels in women[11]. Plasma NPY is derived from sympathetic nerve terminals, and thus levels of NPY in plasma reflect the level of sympathetic activity[12]. Our study was not designed to investigate the regulation of sympathetic activity; therefore, assessments of the effect of the NPY polymorphism on sympathetic tone are warranted.

Methods

Study subjects and analytical methods. Finnish obese subjects: 138 obese subjects (109 women and 29 men) of a weight reduction study[8] with normal liver, kidney and thyroid functions were included in this study. None of the subjects had diabetes, a history of alcohol abuse, major lipid abnormalities or was receiving antihypertensive drug therapy. All phenotype assessments were done by standardized methods in the morning after a 12-hour fast. The parameters assessed included age, weight, height, body mass index (BMI), fat mass, respiratory quotient, basal metabolic rate, waist-to-hip ratio, and systolic and diastolic blood pressure; fasting serum leptin, glucose, insulin, total cholesterol, LDL cholesterol, HDL cholesterol, VLDL cholesterol, and triglyceride levels; and apolipoprotein E phenotypes. Lipoproteins were analyzed after separation by ultracentrifugation. The analytical methods for these parameters have been described[8,13]. Serum leptin was measured by a commercially available RIA kit (Linco Research, St. Charles, Missouri). We also analyzed possible contribution of dietary fat and cholesterol intake to the higher serum cholesterol and LDL cholesterol levels in obese subjects with Pro(7). The diet data were analyzed using a 3-day food diary. The protocol followed the ethical principles of the Helsinki Declaration and was approved by the Ethics Committees of the Universities of Kuopio and Helsinki.

Normal-weight Finnish subjects: Originally a random control population sample, 45–64 years old, was selected during 1979–1981 from the population register of the Kuopio county by using random number tables, taking into account the distribution of the population living in rural and urban communities[14]. Of 144 control individuals, 64 normal-weight subjects (38 women and 26 men) with BMI < 27 kg/m² were selected for this study. They have been regularly followed at 5-year intervals since their recruitment in 1979–1981 (ref. 15). Serum and lipoprotein lipids were determined from 12-hour fasting blood samples. Lipoproteins were analyzed after separation by ultracentrifugation. Serum apolipoprotein B levels were measured only at the 10-year examination. The study population and biochemical methods have been described[14,15]. The Ethics Committee of the University of Kuopio approved the protocol.

Obese and normal-weight Dutch subjects (The Hoorn Study): The Hoorn Study is a prospective follow-up study in a general Dutch population[16]. The study population and research design have been described[16]. 3,553 men and women, 50–75 years old, were randomly selected from the population register of the Dutch town of Hoorn. Of the 2,540 subjects (71.5%) who agreed to participate, 56 non-Caucasians were excluded. Therefore, the study cohort of the Hoorn Study consisted of 2,484 men and women. All subjects gave their written informed consent. The study was approved by

the Ethics Committee of the Vrije Universiteit Academic Hospital. For our study, DNA samples of 263 normal-weight subjects (126 females and 125 males; BMI<27) and 93 obese (60 females and 33 males; BMI >28) were genotyped for the NPY polymorphism. None of the subjects had diabetes or were using antihypertensive or lipid lowering medication at the beginning of the study (1989–1990). Serum total and HDL cholesterol levels were determined from fasting blood samples by enzymatic techniques (Boehringer). The Friedewald formula was used to calculate the level of LDL-cholesterol. Fasting blood glucose levels were determined with a glucose dehydrogenase method (Merck, Darmstadt, Germany). Fasting serum insulin levels were measured by RIA kit (Linco Research, St.Louis, Missouri).

PCR–SSCA, sequencing and genotyping. The PCR primer pairs for amplification of the four exonic areas of the NPY gene were pair 1: 5′–TTGGGGTGTGGGTGGCTC–3′ and 5′–CCTAGACAGACGGGTCGTAGCA–3′; pair 2: 5′–CCCGTCCGTTGAGCCTTCTG–3′ and 5′–CGGTCCCGCGGTCCC–3′; pair 3: 5′–AAAAGACTTTTTTTTTTCCAG–3′ and 5′–AATGTCCCCATCACAAG–3′; and pair 4: 5′–CCTTACATGCTTTGCTTCTTA–3′ and 5′–GATTTTTCATTGAGGAGGAT–3′. Each PCR reaction contained 100 ng genomic DNA (isolated from whole blood), 1.0 mM dNTPs, 30nM ^{33}P-dCTP, 2.5 mM each primer, 0.25 U of AmpliTaq polymerase (Perkin-Elmer, Norwalk, Connecticut). The amplified samples were mixed with SSCA buffer containing 95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromphenol blue (total volume, 25 µl). Of this mixture, 3 µl was loaded on a MDE™ gel (FMC BioProducts, Rockland, Massachusetts). The SSCA gel electrophoresis was run at 5 W constant power in two running conditions: 6 % MDE gel at 4 °C, and 3 % MDE gel with 10% glycerol at room temperature. The abnormally migrating bands in the SSCA were sequenced with the Thermo Cycle Sequenase™ kit (Amersham).

A thymidine(1128)-to-cytosine(1128) substitution in *NPY* generates a *Bsi*EI restriction site, which was used to genotype the subjects for the Leu(7)-to-Pro(7) polymorphism. The PCR products were digested by *Bsi*EI (New England Biolabs, Beverly, Massachusetts) and digestions were analyzed by electrophoresis on 2% agarose gel.

Statistical analyses. The allelic frequency distribution was tested for Hardy-Weinberg equilibrium by the χ^2-test. Statistical differences in phenotype parameters between the two genotype groups were analyzed by Student's *t*-test. Logarithmic transformations were used if data were not normally distributed. Age and sex adjustments for serum lipid parameters were done using the GLM procedure. Additionally, 95% confidence intervals (CI) were calculated for the differences of the mean values between the genotype groups. Because the NPY genotypes were compared with many phenotype parameters in Finnish obese subjects, the Bonferroni-corrected *P* values can be formally obtained by multiplying the presented *P* values (Fig. 1*a*) by the number of comparisons ($n = 14$). In all subsequent analyses (in Dutch obese subjects and Finnish and Dutch normal-weight subjects) we had an *a priori* hypothesis that the Pro(7) allele is associated with serum cholesterol level, and therefore only one dependent variable was originally chosen. The cholesterol data from obese Finns was also analyzed using multivariate analysis of associations between variables and a dichotomic sorting variable (wild-type or mutant) by logistic regression analysis. Univariate analyses with each variable were done first. Stepwise multivariate logistic analyses were then applied on all variables that were significant by univariate analysis. Significant associations were quantified using odds ratios and 95 % CI for them. *P* values less than 0.05 were interpreted as statistically significant. Statistical computations were done using SAS system for Windows release 6.12. (SAS Institute, Cary, North Carolina).

In our study of normal-weight Finns, we analyzed serum lipid levels at three time points during the 10-year follow-up period by analysis of variance (ANOVA) for repeated measurements with two within factors (genotype and time) using the BMDP program 2V (BMDP Statistical Software, Los Angeles, California). When 'pooled' orthogonal components showed non-sphericity, Greenhouse-Geisser adjusted *P* values were used. Because ANOVA for repeated measurements showed a statistically significant influence of the NPY polymorphism on serum total and LDL cholesterol levels, but a nonsignificant effect for time and for the time x genotype interaction indicating a constant effect of the NPY genotype over time, no further analysis was done.

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Neuropeptide Y Polymorphism and Alcohol Consumption in Middle-Aged Men

Jussi Kauhanen,[1,2]* Matti K. Karvonen,[3] Ullamari Pesonen,[3] Markku Koulu,[3] Tomi-Pekka Tuomainen,[2] Matti I.J. Uusitupa,[4] and Jukka T. Salonen[1,2]

[1]*Department of Public Health and General Practice, University of Kuopio, Finland*
[2]*Research Institute of Public Health, University of Kuopio, Finland*
[3]*Department of Pharmacology and Clinical Pharmacology, University of Turku, Finland*
[4]*Department of Clinical Nutrition, University of Kuopio, Finland*

Neuropeptide Y (NPY) plays an important role in the hypothalamic regulation of food intake and energy balance. According to recent findings in animals, NPY also seems to be a potent regulator of alcohol consumption. We used the recently identified Leu(7) to Pro(7) polymorphism in the signal peptide part of NPY to investigate whether the NPY system is associated with alcohol consumption in humans. The subjects (N = 889) were an ethnically homogenous, nonselected population sample of middle-aged men from Eastern Finland. The gene variant producing Pro(7) substitution was associated with a 34% higher average alcohol consumption, even after adjustment for a number of covariates (P = 0.03). The proportion of heavy drinkers (over 230 g of ethanol/week) was also somewhat higher in this group (13.1% vs. 8.2%, P = 0.10). Our study provides the first evidence that alcohol preference in humans is likely to be regulated by the NPY system. Am. J. Med. Genet. 93:117–121, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: alcohol drinking; ethanol; genetic epidemiology; male; neuropeptide Y; polymorphism; population studies

INTRODUCTION

Neuropeptide Y (NPY) is a hexatriocontapeptide amide that is well characterized as a neuromodulator in the central nervous system [Lundberg et al.,1982; Gray and Morley, 1986]. The best known effects of NPY are stimulation of feeding [Clark et al., 1985; Levine and Morley, 1985; Stanley and Leibowitz, 1985] and increased energy storage through lipoprotein lipase activation in white adipose tissue [Billington et al., 1991, 1994]. Recent findings in rodents suggest that NPY may also be a potential regulator of ethanol consumption [Ehlers et al., 1998a,b; Thiele et al., 1998; Cokerill, 1998; Tecott and Heberlein, 1998]. The preference for alcohol seems to be inversely related to NPY levels in brain [Thiele et al., 1998]. NPY-deficient mice show increased consumption of ethanol, whereas transgenic mice that overexpress a NPY gene have a lower preference for ethanol and are more sensitive to its sedative/hypnotic effects [Thiele et al., 1998]. NPY and ethanol have a similar electrophysiological profile [Ehlers et al., 1998b], and both are known to have anxiolytic properties [Heilig et al., 1992; Stewart et al., 1993; Thiele et al., 1998; Palmiter et al., 1998]. In addition, NPY might influence consumption behaviors through reward effects [Ehlers et al., 1998a; Tecott and Heberlein, 1998]. NPY is expressed in the amygdala and nucleus accumbens, structures of the mesolimbic dopamine system that are thought to mediate the rewarding aspects of food, alcohol and certain drugs [Jewett et al., 1992; Ault et al., 1993; Tecott and Heberlein, 1998]. Despite the circumstantial evidence from animal models, no studies on the role of NPY in the regulation of alcohol consumption in humans have yet been published.

A novel finding of a common polymorphism in the signal peptide of NPY was recently reported [Karvonen et al., 1998]. After screening the entire coding region of the NPY gene for sequence variants, a thymidine(1128) to cytosine(1128) polymorphism(*T1128C*) was identified, resulting in a substitution of Leu(7) to Pro(7) in the signal peptide part of preproNPY. The Pro(7) in NPY showed a strong association with elevated serum cholesterol levels [Karvonen et al., 1998].

In the present study our aim was to test the hypothesis that the Leu(7) to Pro(7) polymorphism in NPY is related to the level of alcohol consumption in a nonselected male population sample from the Kuopio Isch-

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*Correspondence to: Jussi Kauhanen, M.D., Ph.D., Senior Research Scientist, the Academy of Finland, Department of Public Health and General Practice, University of Kuopio, P.O.B. 1627, SF-70211 Kuopio, Finland. E-mail: jussi.kauhanen@uku.fi

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emic Heart Disease Risk Factor Study (KIHD) [Salonen, 1988; Lakka et al., 1994].

MATERIALS AND METHODS

Study Subjects

The study population consisted of the participants of the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD), a population-based epidemiologic study that was launched in the 1980s to investigate previously unestablished risk factors for myocardial infarction, progression of atherosclerosis, and other major health outcomes in middle-aged men [Salonen, 1988; Lakka et al., 1994]. The study protocol has been approved by the Research Ethics Committee of the University of Kuopio, and all participants gave a written informed consent to participate in KIHD.

The total sample of the KIHD study consists of 2,682 men who were recruited in two cohorts. The present study is based on the second cohort, that is an age-stratified sample of 42-, 48-, 54-, and 60-year-old men (N = 1,516, participation rate 82.6%) enrolled in the study between 1986 and 1989. A DNA sample was obtained from 1,137 men who were free from coronary heart disease at baseline.

Assessment of Alcohol Consumption

A self-report quantity-frequency questionnaire [Kauhanen et al., 1997a,b] was used to record the level of alcohol use. The average weekly consumption of alcohol in pure ethanol (g/week) was calculated based on the known alcoholic content of each beverage type and the reported doses and frequencies of drinking sessions. We further calculated the proportion of heavy users consisting of those whose average daily consumption exceeded 3 standard doses (>230 g of ethanol/week). One dose is a 12 fl oz bottle of beer, 12 cl of wine, or a 4 cl shot of hard liquor. Serum gamma-glutamyltranspeptidase (GGT) and mean corpuscular volume (MCV) were determined from baseline blood samples as biomarkers of excessive alcohol use. These biochemical measures were checked to see if any of the genotype groups showed biochemical signs of actual alcohol abuse.

Men who stated they had not been drinking at all for at least 12 months were determined as abstainers (N = 123, a total 12.1%). Because abstainers are a heterogenous group consisting of those who have quit because of health problems, they were excluded from final analyses.

Covariates

A number of sociodemographic, behavioral and medical characteristics were assessed according the KIHD protocol as described earlier [Salonen, 1988; Lakka et al., 1994; Kauhanen et al., 1997a]. Age, place of living (urban/rural), marital status, educational level, current income, history of smoking in cigarette-years, and history of diagnosed chronic diseases and conditions (ischemic heart disease, diabetes, stroke, cancer, liver disease, mental disorder) and history of trauma were recorded by a questionnaire and double-checked in the clinical interview. The data were used to examine the possible effect of confounding in the observed relationship.

Genotype Analysis

PreproNPY genotype was determined by restriction fragment length polymorphism (RFLP) analysis from DNA extracted from the subjects' peripheral blood by an investigator unaware of phenotype. Briefly, the polymorphism appears as a thymidine(1128) to cytosine(1128) substitution generating a *Bsi EI* restriction site, that was used to genotype the subjects for the Leu7Pro polymorphism, as described previously [Karvonen et al., 1998]. The PCR products were digested by *Bsi EI* (New England Biolabs, Inc. Beverly, MA) and digestions were analyzed by electrophoresis on 2% agarose gel.

Statistical Analyses

The allelic frequency distribution was tested for Hardy-Weinberg equilibrium by the χ^2-test. Statistical differences in the mean weekly alcohol consumption between the genotype groups were examined in the analysis of variance. Age and other covariates were adjusted for in analysis of covariance. The proportion of heavy drinkers in the genotype groups was compared using a chi-square test. P-values <0.05 obtained from the statistical tests were interpreted as statistically significant. Statistical computations were performed using the SPSS software for IBP RS/6000 (PSS for Unix, SPSS Inc., Chicago, IL).

RESULTS

The analysis of the Leu(7)-to Pro(7) polymorphism in the signal peptide part of the pre-pro-NPY and complete information on alcohol use was available for 889 alcohol using men. Of these, 790 (88.9%) were genotyped as Leu(7)/Leu(7) homozygous, a total of 95 (10.7%) were Leu(7)/Pro(7) heterozygous, and 4 (0.4%) were Pro(7)/Pro(7) homozygous. The allele frequencies were 94.2% (Leu) and 5.8% (Pro). All men carrying either one or two Pro(7) alleles were pooled for further analyses. The study population was in Hardy-Weinberg equilibrium (χ^2 = 0.585, df = 1, P = 0.44).

Table I shows sociodemographic and behavioral background characteristics, and the proportion of men with diagnosed diseases in the two NPY genotype groups. There were no differences in the serum level of gamma glutamyl transpeptidase (GGT) or mean corpuscular volume (MCV) between genotypes. The means and SD of GGT were 29.0 U/l (SD 29.4) among Leu(7)/Leu(7) homozygotes and 29.7 U/l (26.0) among those with Pro(7) (P = 0.83). For MCV the means and SD were 92.0 fl (SD 4.52) and 92.0 fl (SD 4.0), respectively (P = 0.93).

The alcohol consumption in grams of pure ethanol per week is presented in Table II. Both the unadjusted mean consumption and the covariate-adjusted consumption were significantly (33%) higher among men who were carriers of Pro(7). The proportion of heavy drinkers (men who reported drinking on average over 230 g of ethanol/week or over 3 standard doses/day)

TABLE I. Means (SD) and Proportions of Background Variables by the NPY Genotype

	Leu(7) homozygotes (N = 790)	Pro(7) carriers (N = 99)
Age (years)	56.1 (SD 6.7)	56.1 (SD 6.9)
Living in rural area	21.8%	27.0%
Annual income (US $)	24,130 (SD 15,918)	26,862 (SD 14,771)
Educational level (1 = low, 7 = high)	2.05 (SD 1.75)	2.13 (SD 1.92)
Married	87.1%	86.9%
Cigarette smoking (pack-years)	141.3 (SD 292.1)	147.4 (SD 311.7)
Ischemic heart disease	21.1%	13.1%
Diabetes	5.6%	5.1%
History of cancer	2.4%	5.1%
History of stroke	2.6%	1.0%
Liver disease	0.4%	1.0%
History of mental disorder	4.6%	6.1%
History of trauma	10.4%	10.2%

was also higher among men with a Pro(7) substitution (13.1% vs. 8.2%) ($P = 0.10$).

DISCUSSION

We observed an increased alcohol consumption in a population sample of middle-aged men who were homozygous or heterozygous for the variant allele in a common polymorphism substituting Leu(7) by Pro(7) in the signal peptide part of neuropeptide Y (NPY). Presence of Pro(7) was associated with approximately one-third (33%) higher average consumption of ethanol as compared to homozygous subjects with the Leu(7)/Leu(7) genotype. The proportion of heavy consumers who report using over 230 g of ethanol/week was also higher among men with Pro(7) mutation, although this difference did not reach statistical significance due to smaller numbers of subjects.

Our study is the first one to show a relationship between a common NPY polymorphism and alcohol use in humans. The results are in line with the findings from a number of recent animal studies [Ehlers et al., 1998a,b; Thiele et al., 1998; Cockerill, 1998; Tecott and Heberlien, 1998] that have shown an inverse relationship between levels of NPY in central nervous system and preference for alcohol. Mice with no neuropeptide Y are especially fond of alcohol and less sensitive to the effects of ethanol as compared to mice that have normal or extra neuropeptide Y levels [Thiele et al., 1998], and alcohol-preferring rats have lower levels of NPY in amygdala, hippocampus, and frontal cortex [Ehlers et al., 1998a].

The allele frequencies in our study were close to those seen earlier in two Finnish populations [Karvonen et al., 1998]. It is highly unlikely that the observed association could be due to a stratification error in sampling, or population admixture, because Finns are known to be genetically a rather homogenous population.

Many sociodemographic factors are known determinants of alcohol use. In our study the social background among men with and without Pro(7) was similar. The two groups were of the same age and had similar educational background. Slightly more men with Pro(7) were living in rural communities, and this group also had a little higher average income. Smoking history was similar in both groups. It was somewhat unexpected to observe a higher prevalence of ischemic heart disease history among the Leu(7) homozygotes, because earlier findings have shown this genotype to associate with lower serum levels of total and LDL cholesterol [Karvonen et al., 1998]. Adjustment for all these variables in the multivariate model did not affect the observed association between the NPY polymorphism and alcohol consumption, indicating that these variables did not confound the findings.

There are several physiologically plausible mechanisms that can explain the effect of NPY on alcohol use. NPY is an inhibitory neuromodulator that acts widely in the brain. The NPY receptors couple to heterotrimeric G proteins that inhibit production of cyclicAMP [Lamme, 1995; Thiele et al., 1998], so it is possible that NPY inhibits cAMP production in response to alcohol, thus limiting alcohol intake. Central administration of

TABLE II. Mean Weekly Alcohol Consumption in Pure Ethanol According to the NPY Genotype

	Leu(7) homozygotes (N = 790)	Pro(7) carriers (N = 99)	*P*-value
Unadjusted mean alcohol consumption (g/wk)	86.3 (SD 127.6)	115.0 (SD 173.9)	0.030
Mean alcohol consumption (g/wk) adjusted for all covariates[a]	86.4	114.7	0.035

[a]Adjusted for age, place of living, education, income, marital status, smoking history in cigarette-years, history of ischemic heart disease, diabetes, cancer, stroke, liver disease, mental disorder and trauma.

NPY reduces anxiety, and NPY-deficient mice score high on measures of anxiety [Heilig et al., 1992; Palmiter et al., 1998]. The development of alcohol preference may in part depend on the relative lack of tension-reducing NPY.

Chronic exposure to ethanol in rats affects NPY levels in hypothalamus in a fashion similar to food restriction [Ehlers et al., 1998a]. NPY has an important role in the hypothalamic regulation of energy balance by potently stimulating short-term food intake [Clark et al., 1985; Levine and Morley, 1985; Stanley and Leibowitz, 1985]. Centrally administered NPY also increases the expression of lipoprotein lipase mRNA and enhances the enzyme activity in white fat favoring lipid storage [Billington et al., 1991, 1994]. Thus, NPY might nonspecifically affect the consummatory behaviors regarding both food intake and alcohol drinking. There is, however, a lack of NPY transgene expression in the arcuate nucleus of the hypothalamus, a region thought to regulate food intake [Thiele et al., 1998; Palmiter et al., 1998]. This indicates that the effects of NPY on alcohol use are probably not mediated through similar mechanisms as with food and calorie intake.

To our knowledge, there is only one earlier human study examining the possible relationship between neuropeptide Y and addictions. Roy et al. [1990] did not observe significant differences of cerebrospinal fluid (CFS) levels of NPY between male alcoholics and normal controls. Alcoholics, however, do not represent the population at large. It is also unclear, whether the CFS levels of NPY reflect the activity of this peptide in the physiologically important locations of the central nervous system.

Plasma NPY is derived from sympathetic nerve terminals and thus levels of NPY in plasma reflect the level of sympathetic activity [Lundberg et al., 1990]. Significant positive correlations have been observed between levels of NPY and corticotropin-releasing hormone, somatostatin, and growth hormone in cerebrospinal fluid [Roy et al., 1990]. Based on these studies and on our findings, further research on the possible sympathetic nervous system mechanisms in drinking behavior is warranted.

There is no conclusive information of the impact of Pro(7) on the NPY level or function in the central nervous system. Nevertheless, one can speculate that Pro(7) gene variant changes cellular processing of the nascent preproNPY in the endoplasmic reticulum (ER), where proteins fold and oligomerize, disulfide bonds are formed and oligosaccharides are added. The Pro(7) substitution of the Leu(7) could thus lead to changes in the availability or kinetics of NPY release. As seen in animals, the lack of available NPY in the central nervous system associates with heavier consumption of alcohol. Further studies are required to elucidate these mechanisms in detail.

The NPY molecule has already attracted attention as a neuromodulator in the central nervous system and as a regulator of food intake and energy balance. Recent genetic and biochemical studies in animals show that the NPY levels are also related to preference for alcohol. Our genetic epidemiologic study adds further evidence to support the NPY-alcohol hypotheses. It is the first one to suggest there is a link between NPY and alcohol consumption in humans. If replicated in further studies, the findings may have significant clinical and public health implications.

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